THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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FEB 5 1974

Application for Research Grant
(Use extra pages as needed)

Date: Jan. 21, 1974

1. Principal Investigator (give title and degrees):
Anthony J. Triolo, B.Sc., M.Sc., Ph.D.
Associate Professor of Pharmacology

2. Institution & address:
Jefferson Medical College
Thomas Jefferson University
1025 Walnut Street
Philadelphia, Pa. 19107

3. Department(s) where research will be done or collaboration provided:
Departments of Pharmacology & Pathology
1020 Locust Street
Philadelphia, Pa. 19107
Telephone: 215-829-7974

4. Short title of study:
Nicotine on the metabolism and carcinogenesis of benzo(a)pyrene

5. Proposed starting date:
July 1, 1974

6. Estimated time to complete: Approximately 2 years for the nicotine and benzo(a)pyrene studies

7. Brief description of specific research aims:

As part of a collaborative project with the Department of Pathology, this study has the goal of determining whether exposure to low levels of nicotine affects the metabolism and carcinogenicity of benzo(a)pyrene (BP). A/He J mice will be used, since this strain is highly susceptible to BP induced pulmonary and stomach tumors. Some of the fundamental questions that we will attempt to answer as part of our investigation include:

(1) Does chronic feeding for 90 days of nicotine alter the activity of the enzyme BP hydroxylase in lung and stomach and thus influence the tumor incidence at these target organs resulting from the administration of BP?

(2) Can the dietary feeding of nicotine affect the in vitro and in vivo rate of metabolism of BP to form the ultimate carcinogen BP-epoxide?

(3) Are the levels of dietary intake of nicotine and the resulting blood levels of nicotine, as it may enter into the above types of interactions with BP, realistic in terms of the human blood levels of nicotine that are encountered in cigarette smokers?

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8. Brief statement of working hypothesis:

A variety of chemical agents have been shown experimentally to alter the activity of the microsomal enzyme, aryl hydrocarbon hydroxylase (BP-hydroxylase), and thus influence the carcinogenicity of several polycyclic aromatic hydrocarbons (PAH) such as BP. Information is limited, however, on the influence of exposure to nicotine on the metabolism and carcinogenic action of these PAH. We have shown that acute administration of nicotine to rats inhibited the in vivo and in vitro metabolism of BP. The suggested hypothesis of the carcinogenic action of PAH involves their metabolic activation by microsomal enzymes to reactive intermediate epoxides that bind readily to DNA of target organs. Thus, an important aspect of carcinogenesis is the formation of a reactive epoxide, the level of which might be influenced by other chemical agents such as nicotine through its effect on microsomal enzyme activity required for the metabolism of PAH. The accumulation of evidence that prolonged heavy cigarette smoking in humans plays a role in the etiology of cancer of the lung emphasizes the need for more extensive studies of the metabolic and carcinogenic interactions between BP, a known carcinogen, and nicotine, both of which are present in tobacco smoke.

9. Details of experimental design and procedures (append extra pages as necessary)

Experiments will be conducted with female A/He J mice at 9-10 weeks of age obtained from the Jackson Laboratory, Bar Harbor, Maine. Animals will be randomized by weight and placed on experimental diets of Purina Rat Chow to which has been added various levels of nicotine. An illustration of the procedural approach we plan to use in testing the interaction of nicotine on the tumor incidence of BP is as follows: The maximum amount of nicotine in the diet that does not effect the growth rate of rats was reported as 60 ppm (Wilson and De Eds, 1936). Since data are not available to show whether mice will tolerate this level of nicotine, a preliminary pilot study will be conducted in small groups of animals (5 each) to determine the maximum level of nicotine in the diet that does not inhibit growth rate. This will be accomplished by feeding mice for 30 days nicotine at 2-fold increments (15.0, 30.0, 60.0 and 120 ppm) above and below the maximum tolerated dose for rats. Subsequently, mice in groups of 20 each will be fed for 90 days three graded levels of nicotine up to the maximum dose tolerated for 30 days. Corn oil used as a vehicle for the addition of nicotine to the experimental diets will be incorporated in the diet of the control animals.

In accord with the procedure described by Wattenberg (1973), the induction of pulmonary adenoma will be accomplished by administering 3 mg of BP orally on the 4th and 18th day after starting the experimental diet. Forestomach tumors of the mouse will be induced by addition of BP (1000 ppm) in the diet during a 90 day feeding period as reported by Wattenberg (1972). Animals receiving various dietary levels of nicotine alone, and a separate group receiving corn oil in Purina Rat Chow, will serve as controls and both groups will be examined for spontaneous pulmonary and gastric tumors after the termination of the experimental feeding. The following list shows the dietary components of the various groups used in the 90-day feeding study:

#1 Purina chow + corn oil
#2 Purina chow + corn oil + nicotine (x) ppm
#3 Purina chow + corn oil + nicotine (2x) ppm
#4 Purina chow + corn oil + nicotine (4x) ppm
#5 Purina chow + corn oil + nicotine (x) ppm + BP
#6 Purina chow + corn oil + nicotine (2x) ppm + BP
#7 Purina chow + corn oil + nicotine (4x) ppm + BP

Food consumption, nicotine intake and body weight will be monitored weekly during the total period of the experiment, after which the mice will be

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autopsied and their lungs and stomach studied. The number and histologic type of tumors in lung and stomach will be determined, and differences in tumor incidence between control and experimental groups will be expressed by the Carcinogenic Index (the percent of mice with tumors times the mean number of tumors in positive animals).

After termination of the experimental feeding, the in vitro metabolism of BP (e.g., BP-epoxide and hydroxylated products) by liver homogenates from controls (corn oil in Purina Rat Chow) and from animals placed on nicotine diets alone, will be determined by the methods described below. Similarly, blood samples will be obtained before autopsy from all animals on the experimental diet containing nicotine alone, and from animals receiving the diet containing nicotine plus BP, for the analysis of nicotine blood levels by the gas-chromatographic procedure described below.

An attempt will be made to show a correlation between the tissue levels of formed BP-epoxide and any changes observed in the incidence of BP induced tumors. For this purpose, a separate group of feeding experiments will be run to determine the relationship between the dose of nicotine and the in vitro and in vivo formation of BP-epoxide derived from BP by the methods described below.

In vitro Metabolism of BP

The activity of BP hydroxylase will be determined in various tissues. The method to be used involves the measurement of fluorescence in an Aminco-Bowman spectrophotofluorometer of hydroxylated metabolites of BP by the general procedure of Dehnen et al. (1973). Since a problem may exist in determining low levels of BP hydroxylase activity in some tissues (e.g. forestomach), the method described by Dehnen et al. (1973) was selected because it is more sensitive for this assay and extraction of metabolites with organic solvents is not required as in other available methods. The determination of BP hydroxylase activity in tissue with high activity, such as liver, is also important, because changes in enzymatic activity at this site by exposure to nicotine and BP may alter the tissue levels of BP and its metabolites in target organs (e.g. lung and forestomach) that are susceptible to BP induced tumors. Enzyme activity will be expressed as the quantity of reference standard 3-hydroxy-BP causing fluorescence equivalent to total hydroxylated metabolites produced per mg of tissue during the time of incubation. Optimal conditions for all enzyme reactions will be determined by testing various incubation times and enzyme and substrate concentrations.

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For the analysis of BP-epoxide formed in vitro by incubating tissue homogenates with BP, the method used will be that of Grover et al. (1972). This procedure requires the incubation of liver microsomes equivalent to 10 gm liver with cofactors required for an NADPH-generating system, and pre-incubating (30°C for 10 min) with 1,2 epoxy-1,2,3,4-tetrahydronaphthalene, to increase the yield of epoxides by inhibiting the enzyme-catalyzed (by epoxide hydrase) conversion of epoxides to dihydrodiols. The reaction is initiated by the addition of tritiated BP (400 ugm), and after incubation the residue of an ether extract is chromatographed on an alumina column and partitioned into fractions of BP, BP-epoxide and hydroxylated products. Though application of this method to tissue with low enzyme activity (e.g. lung and forestomach) may be difficult, an attempt will be

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made to determine the in vitro metabolism of BP to its epoxide in these tissues from controls and animals receiving various dietary levels of nicotine. In similar experiments an attempt will also be made to measure the tissue level of BP-epoxide in vivo derived from the intravenous administration of BP either during the dietary feeding of nicotine or shortly after the 90-day feeding period.

Blood Levels of Nicotine

Animals will be bled by cervical incision after dietary feeding of nicotine and the blood will be collected in heparinized tubes. Nicotine will be analyzed according to the procedure described by Isaac and Rand, 1972. After centrifugation of blood the internal standard modaline is added to plasma which is then alkalinized and extracted with diethyl ether. The ether extract is reduced to an appropriate volume and 2.0 ul are injected into the gas chromatograph. A Hewlett-Packard model 5750B gas chromatograph fitted with an alkaline flame ionization detector will be used to analyze plasma extracts of nicotine.

Statistics

Only probability values of 5% or less will be considered significant for all data obtained. All experiments will be designed so that the results may be subjected to statistical analysis using, when appropriate, student t-test and coefficient of correlation.

References

1. Dehnen, W., Tomingas, R. and Roos, J. A modified method for the assay of benzo(a)pyrene hydroxylase. Anal. Biochem. 53: 373, 1973.

2. Grover, P.L., Hewer, A. and Sims, P. Formation of K-region epoxides as microsomal metabolites of pyrene and benzo(a)pyrene. Biochem. Pharmacol. 21: 2713, 1972.

3. Isaac, P.F. and Rand, M.J. Cigarette smoking and plasma levels of nicotine. Nature 236: 308, 1972.

4. Wattenberg, L.W. Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic anti-oxidants and ethoxyquin. J. Natl. Cancer Inst. 48: 1425, 1972.

5. Wattenberg, L.W. Inhibition of chemical carcinogen-induced pulmonary neoplasia by butylated hydroxyanisole. J. Natl. Cancer Inst. 50: 1541, 1973.

6. Wilson, R.H. and DeEds, F. Chronic nicotine toxicity: I. Feeding of nicotine sulfate, tannate and bentonite. J. Indust. Hyg. Toxicol. 18: 553, 1936.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities for this project include three fully-furnished research laboratories throughout the year and an adjacent student laboratory for nine months of the year. Adequate animal rooms and caging are available within the department, and we also have use of an institutional central animal quarters. Workers on this project have access to departmental stocks of standard chemicals, drugs, glassware, apparatus and instruments for routine toxicologic and pharmacologic experimentation.

Equipment now located in the three "central base" laboratories include: 2 fume hoods, 1 Labline incubator, 2 Eberbach water bath shakers, 1 torsion and 2 roller Smith Precision Balances for tissue weighing, 2 pH meters, 2 high speed refrigerated centrifuges (International and Sorvall), 1 F&M Gas Chromatograph with flame ionization, 1 Hewlett-Packard Gas Chromatograph, flame ionization and electron capture detectors, 4 Vortex mixers, 2 hot air drying ovens, 3 hot plates, 3 timers, 1 Gilford spectrophotometer, and 2 Bausch and Lomb Spectronic 20 colorimeters. Specialized equipment located elsewhere in the department but available for this project include cold rooms (refrigerator and freezer), incubator room, 2 Spinco Model L ultracentrifuges, 1 Perkin Elmer infrared spectrophotometer, 2 Packard liquid scintillation spectrometers. 1 Aminco-Bowman spectrophotofluorometer, and 1 Aminco Chance Dual Wavelength/Split Beam Spectrophotometer.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Pages 6 through 15 of grant proposal

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

(See page 3-A)

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13. A. Manuscript accepted in Science pertinent to this grant proposal is appended to application (page 16).

B. Five (5) publications appended to application (page 17).

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Anthony J. Triolo (Princip. Invest.)	30	$ 6,270
Julius M. Coon (Co-Princip. Invest.)	15	5,760
Gonzalo E. Aponte (Co-Princip. Invest.)	5	3,000
Post-Doctorate (to be recruited)	100	11,000
Technical		
Research Technician (to be recruited)	50	4,000
Fringe benefits on above (13%)		3,903
	Sub-Total for A	33,933

B. Consumable supplies (by major categories)	
Animal, animal care	2,500
Prepared purina diets	1,000
Chemicals & cofactors	1,500
Glassware & misc.	1,000
Sub-Total for B	6,000

C. Other expenses (itemize)	
Equipment maintenance & repair	800
Laundry	200
Travel to scientific meetings	600
Sub-Total for C	1,600
Running Total of A + B + C	41,533

D. Permanent equipment (itemize)

High sensitivity nitrogen detector
Model #1516B Hewlett-Packard
for plasma analysis of nicotine

Sub-Total for D	730
E	6,230
Total request	48,493

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$35,968	$6,300	$1,600	--	$6,580	$50,448
Year 3	30,484	6,615	1,600	--	5,805	44,504

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16. Other sources of financial support: None

List financial support from all sources, including own institution, for this and related research projects.

Approximately $1000 available from the Department of Pharmacology operating budget

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	None		

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Organophosphates on benzo(a)pyrene carcinogenesis	National Institute of Health Submitted Jan., 1974	$179,744	Sept. 1, 1974 to Aug. 31, 1979

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Anthony J. Triolo, Ph.D.

Signature Anthony J. Triolo Date Jan. 22, 1974

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Responsible officer of institution

Typed Name William F. Kellow, M.D.

Title Dean & Vice President

Signature William F. Kellow Date 1/28/74

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